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STUDIES OF THE ENZYMATIC DEAMINATION OF CYTOSINE ARABINOSIDE—II

PROPERTIES OF THE DEAMINASE OF HUMAN LIVER

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Abstract—A sensitive, radioisotopic assay for the pyrimidine nucleoside deaminase present in human liver was developed. Uracil arabinoside was the only product detected after incubation of cytosine arabinoside with a crude homogenate of human liver. The deaminase was stable at elevated temperatures and after prolonged storage under refrigeration. No cofactor requirements were demonstrated, but enzyme activity appeared to depend upon the presence of reduced sulfhydryl groups. The K_m value for cytosine arabinoside was $1{\cdot}2$–$1{\cdot}6 \times 10^{-4}$M. No *direct* mechanism for regulating deaminase activity was detected.

IN A PREVIOUS communication,[1] Camiener and Smith reported on the discovery and preliminary characterization of a pyrimidine nucleoside deaminase in human liver. This enzyme could be responsible for the rapid degradation of cytosine arabinoside* which occurs in man,[2–4] and this prompted studies aimed at partially characterizing the enzyme and determining whether its effects could be negated. The present paper describes some of the properties of the deaminase of human liver and the radioisotopic procedure that was used for the assay of its activity. The following paper[5] presents some of the substrate requirements of the enzyme and its inhibition by a variety of compounds. A preliminary account of part of these studies has appeared elsewhere.[6] Most of the work has been done with crude homogenates of whole liver since the primary goal has been to obtain deaminase inhibitors capable of inhibiting the enzyme *in vivo*. Little attention has been given to enzyme purification except where it would contribute to this primary goal.

MATERIALS AND METHODS

Materials

The preparation of human liver homogenates and the composition of KR buffer have been described earlier.[1] CA† and UA were prepared in the Upjohn laboratories. CA-^{3}H, UA-^{3}H, XaR, and dCMP were obtained from Schwarz BioResearch Inc., Orangeburg, N.Y. C, U, G, R, and A were purchased from Nutritional Biochemicals Co., Cleveland, Ohio. dUMP, dCDP, and TDP were products of Sigma Chemical Co.,

* 1-β-D-arabinofuranosylcytosine (generic name, cytarabine)

† The abbreviations used are: Ad for adenine; C for cytosine; G for guanine; Hx for hypoxanthine; U for uracil; Xa for xanthine; A for d-arabinofuranose; R for D-ribofuranose; dR for 2′-deoxy-R; CR, TdR, etc., for cytidine, thymidine, and other 1-β-D-sugar pyrimidines; GMP, UDP, CTP, etc., for 5′-ribonucleotides; dAMP, dCDP, TTP, etc., for 5′-deoxyribonucleotides.

St. Louis, Mo. All other pyrimidine and purine-containing compounds were purchased from Calbiochem, Los Angles, Calif.

Assays

Spectrophotometric, cytotoxic, and paper chromatographic assays have been presented elsewhere.[1] In the radioisotopic assay, enzyme reaction mixtures containing tritiated CA or CR were stopped with 0·5 vol. of iced, 15% (w/w) trichloroacetic acid, and the precipitates were removed by centrifugation. Two 2-μl quantities of the clear supernatant solution were co-chromatographed with a mixture containing 40 mμmole each of nonradioactive carrier materials on Whatman no. 40 paper. Efforts were made to keep the spots as small as possible. The papergrams were developed by the descending technique for 16 hr at room temperature with an isopropanol: conc. HCl:water solvent system (170 ml:41 ml:water to 250 ml).[7] After drying, the substrate and product zones were located under u.v. light, and these zones (including a 2–4 mm extra margin) were cut into small pieces and placed in scintillation vials. The pieces of paper were wetted with 0·25-ml aliquots of distilled water per vial, 15-ml portions of Diotol[8] were added, and radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. In a micromodification of this procedure, aliquots (0·05–0·10 ml) of the enzyme reaction mixtures were transferred at appropriate times to Seditubes,* and the samples were processed in the manner just described. The R_f values for the nucleosides in this solvent system were as follows: UA, 0·74; UR, 0·66; CA, 0·57; CR, 0·47. To minimize errors arising from sample preparation, dilution, and micropipetting, the assay results were calculated, in most instances, as the per cent (rather than the absolute amount) of substrate (CA) converted to product (UA).

Purification of CA-^3H

Solutions of highly tritiated CA are frequently contaminated to some extent with a variety of degradation products. In most experiments these mixtures were used without purification, since it was possible to make valid calculations by using appropriate controls. When required, however, chromatographically pure material can be prepared by descending chromatography on Whatman no. 40 paper at room temperature in a solvent system consisting of isobutyric acid:1 M NH_4OH:0·1 M EDTA,† 500 ml: 300 ml:8 ml.[9] The R_f values for CA, CR, and some of their breakdown products in this system are: C, 0·84; CA, 0·74; UA, 0·54; A, 0·42; CR, 0·75; UR, 0·56; R, 0·45. Two unidentified materials, always present in trace amounts, have R_f values of 0·35 and 0·26 respectively. U, which thus far has not been detected in breakdown mixtures, has an R_f value of 0·63.

EXPERIMENTAL AND RESULTS

Radioisotopic assay

Statistical analysis of the radioisotopic assay data showed good reproducibility throughout. On single chromatography sheets, the zone-to-zone reproducibility (after development in solvent) showed standard deviations ranging from 0·4 to 1·6 per cent at average cpm values of 4000; sheet-to-sheet variations ranged up to a

* Seditube, Becton, Dickinson & Co., Rutherford, N.J.

† The presence of EDTA reduces streaking.

maximum standard deviation of 1·7 per cent. Similarly, the sum of background-corrected cpm values for substrate zones (CA or CR) plus those of product zones (UA or UR) was found to be quite constant. In a random survey of 27 separate experiments, each having an assay at four different time intervals, the standard deviations of the four sums ranged from 2·6 to 5·8 per cent with an average value of 4·1 per cent; total cpm values in these experiments ranged from 1000 to 4000. The assay reproducibility was attributable, in large measure, to the spotting of small amounts of sample. Attempts to increase sample sizes from 4 μl to 10 or 20 μl resulted in appreciable streaking of the zones.

The elution of the tritiated nucleosides from the paper into the Diotol was complete in less than 4 hr (the shortest time period checked). It was found that the paper in the vials must be present as relatively small pieces; counting efficiencies decreased as the size of the paper became larger. This decrease was found to result from physical interference with the photocell detection of scintillations. In control vials, the presence of a single 2·5-cm diameter circle of paper reduced counting efficiencies by 11 per cent; a 1·3-cm diameter circle effected a 5 per cent decrease.

No significant degradation of either CA, CR, UA, or UR could be demonstrated after standing in 5% trichloroacetic acid for several hours at 0°. Similarly, no significant change could be demonstrated during the overnight chromatography step.

Enzyme reaction products

UA was the only product of the CA deamination reaction detected with the human liver homogenate (Table 1). If other products were formed, they would have to have

TABLE 1. THE PRODUCTS OF THE REACTION OF HUMAN LIVER HOMOGENATE ON CA AS A FUNCTION OF INCUBATION TIME*

Incubation time (min)	Total cpm recovered	Per cent of total nucleoside†	
		CA	UA
0	32,700	100	0
5	33,100	89·5	10·5
15	33,100	63·9	36·1
45	34,100	16·2	83·8

* The incubation mixture, which was prepared in a 12-ml centrifuge tube in an ice bath, contained 0·50 ml glycylglycine buffer (1·25 M, pH 8·0), 0·14 ml CA-^{3}H (2·5 μmole, 35 μc/mole), 0·50 ml of a centrifuged 25% homogenate of normal human liver prepared in KR buffer, and sterile distilled water to a final volume of 1·25 ml. After 0, 5, 15, and 45 min of incubation at 37°, 0·2 ml-aliquots were removed, chilled in an ice bath, and processed as described in Methods. The solvent system used was the isobutyric acid system described in the text. After drying, the strips (3/4 in. wide) were cut into 1/8-in. rectangles parallel to the solvent front, and the pieces were placed in scintillation vials containing 14·75 ml Diotol plus 0·25 ml water. Twenty-four hr and several mixings later, the samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. Counting efficiences were approximately 8–9 per cent.

† The results have been corrected for non-CA material present in the zero-time control.

been present in amounts less than 0·2 per cent of the total radioisotope, the limits of detection in this study.

Enzyme stability

No losses of enzyme activity were detected in either homogenates of human liver stored in the freezing compartment of a laboratory refrigerator for 6 months, or pieces of human liver stored at liquid nitrogen temperatures (−196°) for more than 2 yr.

The effects of incubation temperatures and exposure times on the enzymatic deamination of CA were studied in a series of three experiments. The results of these studies were plotted in a form corresponding to the Arrhenius equation (Fig. 1). A confirmatory study showed a linear relationship between the rate of deamination and 1/T at all temperatures below 75° that were studied.

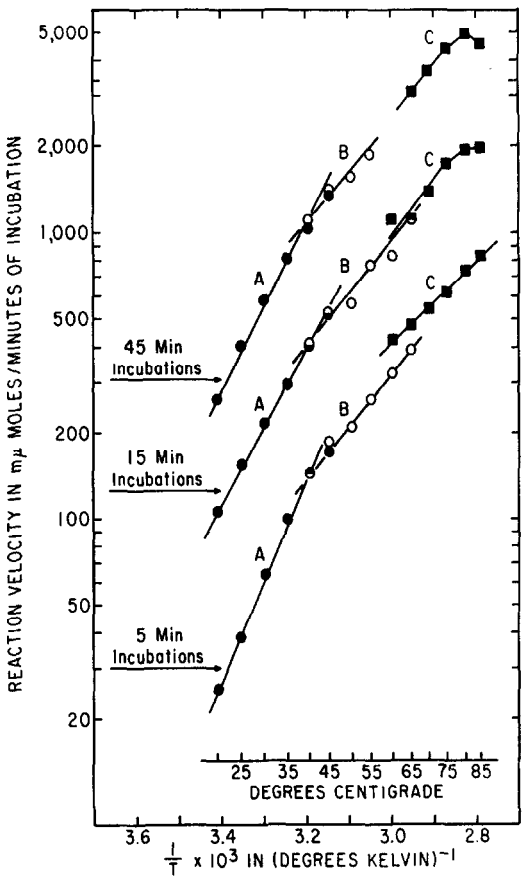

FIG. 1. The effects of incubation temperatures and time on the enzymatic deamination of CA. The results of 3 separate experiments (Curves A, B, and C) have been pooled in this single figure. The incubation mixtures were prepared as described in Table 2 and incubations were at the temperatures shown in the figure. At intervals of 5, 15, and 45 min, aliquots of 0·05–0·10 ml were removed for assay. For temperatures of 20° – 65°, the reaction mixtures contained 0·1 ml CA (2·5 μmole, 1 μc/μmole); for 60° – 85°, the mixture contained 0·15 ml CA (7·25 μmole, 0·67 μc/mole).

The unusually high temperature stability of the deaminase permitted the use of a heat step for the purification of the enzyme. The optimum conditions called for the incubation of a centrifuged, 25% homogenate of normal human liver for 5 min at 80° in a circulating water bath. The reaction was stopped by chilling in an ice bath, and precipitate was removed by centrifugation at 1000 *g* for 15 min at 4°. This purification step (Table 2) provided a 6-fold purification of the deaminase and, at the same time, probably inactivated most other enzyme activities. It was this last aspect that allowed the demonstration that two enzymes were involved in the deamination of CMP and dCMP.[5]

TABLE 2. THE EFFECT OF HEAT ON THE PURIFICATION OF THE DEAMINASE

Homogenate treatment*	UA product formed† (mμmole)	
	Per ml homogenate	Per mg protein‡
None	2200	65
75°, 15 min	1850	290
80°, 5 min	1740	390
80°, 15 min	1030	340

* See the text.

† The incubation mixtures, which were prepared in 12-ml centrifuge tubes in an ice bath contained 250 μmole glycylglycine buffer at pH 8·0, 1·0 μmole CA-^{3}H (5 μc/μmole), 0·2 ml of a centrifuged homogenate of normal human liver prepared in KR buffer, and distilled water to a total volume of 0·5 ml. The tubes were incubated at 37° for 30 min, and the tube contents were assayed as described in Methods.

‡ Protein was determined by the method of Oyama and Eagle.[10]

Check for allosteric regulation

The allosteric regulation of an enzyme activity can be demonstrated in one of two ways, either by removing the allosteric cofactor from its associated enzyme by various procedures such as dialysis, or by adding the cofactor to a cofactor-free preparation. Further, the need for cofactor often becomes more pronounced as the enzyme is purified.[11] In dialysis experiments with both crude and partially purified (i.e. heat-treated) deaminase preparations, no changes in activity were observed even when the preparations were dialyzed for 16–20 hr at 4° against 100–1000 vol. of KR buffer or 0·9% saline containing 10 g Darco G-60 activated carbon/liter. Similarly, no significant change in activity was observed when a variety of purines, pyrimidines, and their nucleosides and nucleotides* was added, in 1 μmole amounts, to reaction mixtures (Table 2) containing extensively dialyzed (and presumably cofactor-free) enzyme preparations. Thus, it was concluded that the pyrimidine nucleoside deaminase did not appear to be subject, in our tests, to an allosteric regulatory mechanism.

* The compounds tested were UMP, UDP, UTP, dUMP, TMP, TDP, TTP, CMP, CDP, CTP, dCMP, dCDP, dCTP, Ad, Ad-R, AMP, ADP, ATP, Ad-dR, dAMP, dADP, dATP, G, GR, GMP, GTP, GdR, dGMP, dGDP, dGTP, Hx, HxR, Xa, and XaR. Nucleoside substrates and reaction products of the enzyme were not tested in this study. Data for these compounds are presented elsewhere.[5]

Kinetics of the deamination

The K_m and V_{max} constants for CA were determined by the double reciprocal plot method of Lineweaver and Burk;[12] the values measured in Fig. 2 were $1{\cdot}2 \times 10^{-4}$ M and 0·43 μmole/min/g liver wet weight respectively. K_m and V_{max} constants from repeat experiments were in close agreement with the above values. They ranged from $1{\cdot}2$ to $1{\cdot}6 \times 10^{-4}$ M, and from 0·40 to 0·45 μmole/min/g liver wet weight respectively.

Other enzyme properties

The deaminase did not appear to require any cofactors for activity: (a) dialysis experiments were reported above; (b) no loss of activity was observed when the

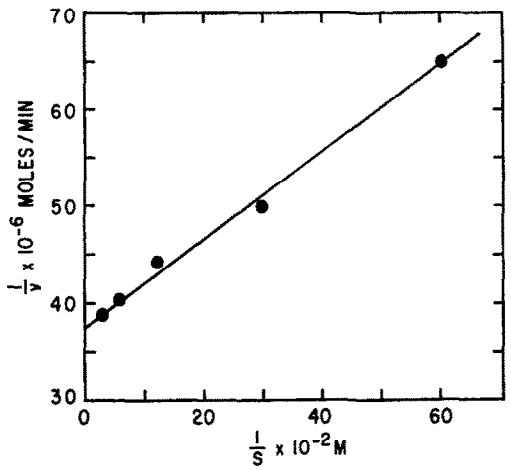

FIG. 2. The determination of K_m and V_{max} constants for the deamination of CA by human liver deaminase. Reaction mixtures and incubation conditions were those described in Table 2. The substrate, CA-^{3}H (5 μ/tube), was present in the reaction mixtures at the indicated concentrations. The reaction velocity for each substrate concentration was calculated from the initial linear portion of each deamination curve.

homogenate was preincubated with EDTA, 10^{-4} M (Table 3); and (c) no loss of activity was observed when the enzyme was preincubated (37°, 30 min) with semicarbazide hydrochloride, 10^{-4} M.

Deaminase activity did appear to depend, however, upon the presence of easily oxidized sulfhydryl groups, as shown in Table 3. Preincubation of the homogenate with *p*-chloromercuribenzoate destroyed deaminase activity; preincubation with EDTA, 10^{-3} M, slightly enhanced activity. Reduced glutathione and mercaptoethanol had no effect on enzyme activity at concentrations of 10^{-3} M, but at 10^{-2} M these compounds markedly inhibited the enzyme. This inhibition is believed to have resulted from the formation of mixed disulfides of the compounds with the enzyme.

Buffer experiments performed previously[1] with the spectrophotometric assay were rechecked with the radioisotopic assay; again, the same broad pH optimum between pH 7 and 10 was observed.

TABLE 3. THE ROLE OF SULFHYDRYL GROUPS IN THE ENZYMATIC DEAMINATION OF CA*

Test compound	UA-^{3}H formed		
	Amount	mμmole/tube	% Inhibition
None (water)	—	610±19†	0±3·1†
p-Chloromercuribenzoate	10^{-4} M	<20	>97
	10^{-5} M	49	92
	10^{-6} M	630	0
EDTA	10^{-3} M	690	13% stimulation
Reduced glutathione	10^{-2} M	<20	>97
	10^{-3} M	620	0
Reduced mercaptoethanol	10^{-2} M	200	67
	10^{-3} M	570	7

* The components of the reaction mixture are described in Table 2. Test compounds were preincubated in 12-ml centrifuge tubes for 30 min at 37° with buffer and liver homogenate in a total volume of 0·5 ml. The tubes were placed in an ice bath, and 0·1-ml aliquots of CA-^{3}H substrate solution (10 μmole/ml, 5 μc/μmole) were added to all tubes. The tubes were incubated for 45 min at 37°, and the tube contents were assayed as described in Methods.

† Mean ± 1 S.D.

DISCUSSION

The HCl–isopropanol solvent used in the radioisotopic assay was selected for two reasons. First, all radioactivity (including CA and UA degradation products present in unpurified isotope solutions, see Methods) moved on the chromatograms with either the CA or UA; this made it easy to determine the actual conversion of CA to UA. Second, the UA zone moved faster than that of CA in this solvent system, and this minimized 'tailing' problems associated with the measurement of small amounts of product in the presence of large amounts of substrate. This latter situation is encountered when the initial rates of an enzyme reaction are being determined. The isobutyric acid solvent system, used earlier to characterize the products of the reaction, was not suitable for routine assay purposes since it separated the reaction mixtures into too many zones.

The nucleoside deaminase described here has many properties in common with the nucleotide (dCMP) deaminase described by Maley and Maley. Both enzymes (a) are present in mammalian tissues,[1, 13] (b) appear to be simple hydrolytic deaminases,[1, 14] (c) are sulfhydryl-dependent,[14–16] (d) have fairly specific substrate requirements,[5, 14] and (e) are inhibited to about the same degree by analogous inhibitors.[5, 14, 16] It therefore became important to compare other properties of these deaminases in order to see how far the similarities continued.

One such property that required investigation was the regulation of dCMP deaminase activity by TTP and dCTP. Maley and Maley have demonstrated the effects of these allosteric cofactors on the stability of the enzyme when present in crude homogenates;[11, 17, 18] they showed the more-pronounced effects of these cofactors, as well as those of some purine nucleotides, in dialyzed and partially purified enzyme preparations.[11, 15, 16] Similar experiments with our nucleoside deaminase were not

successful in demonstrating a comparable enzyme regulation. No loss in deaminase activity was observed when either crude or partially purified enzyme preparations were subjected to extensive dialysis; no inhibition or stimulation of deaminase activity was seen when various purine and pyrimidine bases, nucleosides, and nucleotides, were added to dialyzed enzyme preparations. It would thus appear that the two deaminases are subject to different regulatory mechanisms, if in fact the nucleoside deaminase is subject to any direct regulatory mechanism.

The direct regulation of enzyme activity must occur at one of two sites, either at the active site of the enzyme (as in competition between substrates or inhibition by products) or at an allosteric site. Attempts to demonstrate a direct regulatory mechanism for the pyrimidine nucleoside deaminase have not been successful to date. Competition between multiple substrates would seem to be ruled out by the very specific substrate requirements of the enzyme;[5] product inhibition would appear to be eliminated by the relative insensitivity of the enzyme to large UA:CA ratios;[1] allosteric regulation is unlikely in view of the preceding data and discussion. On the other hand, it is difficult to conceive of a potent and prominent enzyme activity that is not subject to some sort of direct regulation.

Comparison of the properties of the human liver deaminase with those reported by Creasey[19] for mouse kidney deaminase showed several interesting differences and similarities. Both enzymes were dependent upon the presence of free sulfhydryl groups for activity, but the mouse deaminase, in addition, rapidly lost activity unless stored with sulfhydryl-protective materials such as reduced glutathione. Both enzymes appeared to be simple hydrolytic deaminases[1] and both were specific in their substrate requirements.[5] The human liver deaminase appeared to have a slightly stronger affinity for both the CA and CR substrates than did the mouse kidney deaminase. Creasey reported a K_m value for CR of $2{\cdot}1 \times 10^{-4}$ M and, based on his reported CA/CR ratio of 0·06, one would predict a K_m value for CA of $\geqslant 2 \times 10^{-4}$ M. In the study reported here, CA had a K_m value of about $1{\cdot}4 \times 10^{-4}$ M and, based upon the competitive efficacy and deamination data,[4] one would predict a K_m values for CR of $<1 \times 10^{-4}$ M.

The enzyme studies have shown that the pyrimidine nucleoside deaminase was present in relatively large amounts in human liver, was specific in its substrate requirements,[5] was active over a broad pH range, was heat stable, and was subject to no known direct regulatory mechanism.

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